*DIABETES MELLITUS AND AGING: DIMINISHED PLATING EFFICIENCY OF CULTURED HUMAN FIBROBLASTS**

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Communicated by Herman M. Kalckar, July 7, 1969

Abstract.—This work concerns the effect of age and the diabetic gene(s) on the growth capacity of skin fibroblasts in culture. Cells from normal subjects and the progeny of conjugal diabetics have similar lifespans after multiple passages in mass culture. The combined lifespans *in vitro* are inversely proportional to the age of the donor. When individual cells are plated, more of those from normal subjects are able to form colonies. The difference in plating efficiency is apparent when first tested after 20 generations of growth, persists at 30 and 40 generations, but disappears after 50 generations. This cell culture system should be useful in studying the inheritance of the diabetic gene(s), the pathogenesis of the diabetic state, and the relationship between aging and diabetes, both of which decrease plating efficiency.

Introduction.—The potential to develop diabetes mellitus is inherited, but the precise nature of genetic transmission is a matter of controversy.[1] Recently, opinion has favored a polygenic pattern of inheritance.[2, 3] However, for expression of the disease, an unexplained interaction with other factors is required, particularly with the influences of aging.[4, 5] Thus, in prediabetics (defined as the offspring of conjugal diabetics or the unaffected monozygotic twin of a diabetic) the incidence of overt diabetes is about 50 per cent at age 40 and only begins to approach 100 per cent long after this time.[6–8]

The cultured diploid fibroblast has several advantages as a model system in seeking a cellular abnormality in this disease. It provides an opportunity to study the genotype of cell populations in a controlled environment, several generations removed from the neurohumoral influences of the donor. It allows the direct study of spontaneous diabetes of man, rather than experimental systems in animals. Finally, it is a model for the study of aging *in vitro*, since these cells have a finite lifetime on repeated subculture; after a period of vigorous growth they undergo senescence and eventually die.[9, 10] Furthermore, their potential to propagate *in vitro* seems to be related to the age of the donor *in vivo*.[10, 11] In this regard, physiological rather than chronological age seems to be important, because fibroblasts from patients with Werner's syndrome[12] and progeria,[13] diseases associated with premature aging and diabetes, have a shortened lifespan when cultured *in vitro*.

Earlier studies[14] comparing cultured fibroblasts from diabetics and normal individuals failed to reveal significant differences in glucose metabolism, either basal or stimulated with insulin. Explanations offered included the possibility that the effect of the diabetic gene(s) was not directly related to carbohydrate metabolism

in fibroblasts, or that cells derived from diabetic skin explants had undergone selection so that only the "hardiest" cells grew to be assayed. To circumvent these possibilities, it seemed desirable to study the pathogenesis of diabetes by examining another parameter in cells from a different set of subjects. This report deals with the growth capacity of cultured fibroblasts from a group of individuals not yet showing the overt disease, but carrying a high genetic risk to do so.

Materials and Methods.—Subjects: All the high-risk subjects in this study were the offspring of two clinically overt diabetics. This group was clinically normal but was subdivided into two categories:[15] (1) prediabetics, with repeatedly normal intravenous and oral glucose tolerance tests, the latter with and without cortisone augmentation, and (2) chemical diabetics, with fasting euglycemia, but with one or more abnormal tests. Normal controls were of the same age group, had negative family histories for diabetes, and were consistently found to have normal glucose tolerance. Detailed information on individual patients is available upon request.

Cell culture: Techniques have been described previously.[14] In brief, all cultures were derived from skin biopsies of the forearm explanted into Eagle's minimum essential medium plus 15% fetal calf serum. After approximately 4 weeks of incubation at 37°C in a humidified atmosphere of 95% air:5% CO_2, fibroblasts growing from the explants were released with trypsin and transferred to large Petri dishes for further culture. Plating efficiency experiments were performed according to the method of Ham and Puck,[16] and as described in detail in the legend to Figure 2. Plating efficiency equals 100 times the mean number of colonies divided by the number of cells inoculated. Passage of mass cultures was accomplished by using a 1:8 subcultivation ratio. In this way it is estimated that each cell divides approximately three times before contact inhibition arrests further multiplication.[10] All dishes were inspected microscopically each day, and cells were subcultured immediately after becoming confluent. Toward the end of the lifespan, cell growth slowed markedly in spite of frequent replacement of the growth medium. A strain was "pronounced dead" when its cells became swollen and granular and failed to grow to confluence despite repeated feeding.[10] After they were pronounced dead, seven controls and seven high-risk strains were examined for mycoplasma, with negative results.

Results.—The lifespans of control and high-risk cells in mass culture are shown in Figure 1. The two groups were statistically indistinguishable. If number 6 is omitted, the combined data of both groups showed an inverse correlation between age of donor and lifespan *in vitro* ($p < 0.01$), confirming earlier studies.[10, 11] The time span of the initial explant stage averaged four weeks for the controls and the same for the high-risk group, with a range of ±one week in each group; the number of cell generations during this period is unknown but has been set arbitrarily at ten.

In mass culture "hardy" cells may assist "weak" cells by an indirect exchange of metabolites or macromolecules,[17] direct exchange of cytoplasmic material,[18] or even by cell fusion.[19] Since these phenomena could obscure strain differences, experiments were undertaken to quantitate the ability of individual cells to attach and form colonies.

Figure 2 shows that control cells plated with a mean efficiency of 12.1% ± 1.6 SEM, which is in the range reported by other investigators.[20] The high-risk group plated with a mean efficiency of 7.0% ± 0.5. Though the distributions of the two groups overlap, the difference between their means is highly significant ($p < 0.002$). This is true even if normal subjects nos. 19 and 23 are omitted from the calculation ($p < 0.005$). No correlation was found between plating efficiency and age of donor or glucose tolerance in the high-risk group; nor did severity of

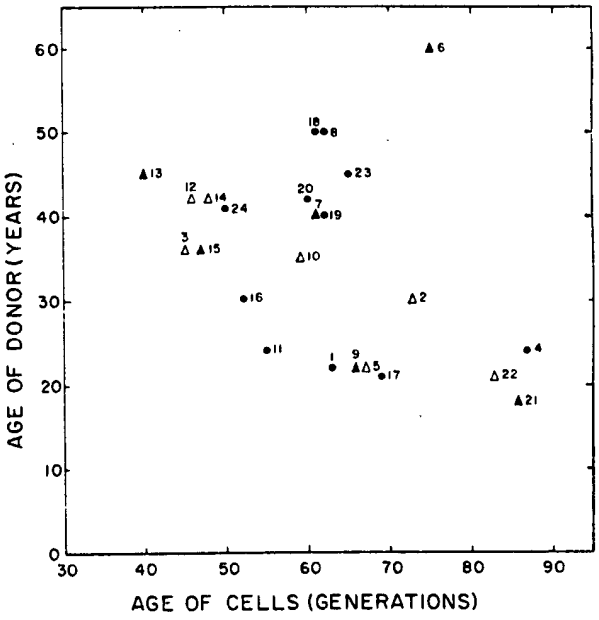

FIG. 1.—Relationship of age of normal controls (●), high-risk prediabetic (▲), and chemical diabetic (△) donors to the lifespan of fibroblasts grown *in vitro* in mass culture. The number of cell generations used up by the primary explants in attaining the first confluent monolayer has been arbitrarily set at ten, as mentioned in the text. The index numbers of subjects are shown. Each value is the average of two separate lifespan determinations, which agreed (±10%) within each strain. Exceptions were nos. 21 and 22 for which the second lifespan determination was about twice as long as the first; the greater value is plotted, without averaging. High-risk no. 6 is a prediabetic with consistently normal glucose tolerance; his father was insulin dependent and died at 73, while his mother required tolbutamide and died at age 83.

diabetes in the parents (scored by factors such as age of onset, insulin-dependency, complications, etc.) correlate with the plating efficiency of cells derived from offspring.

Figure 3 shows that the mean plating efficiencies of the normal and high-risk strains were significantly different again at 30 ($p < 0.01$) and 40 generations ($p < 0.05$), but this was no longer true at 50 generations. Several strains were "dead" by 50 generations, and not used for plating efficiency determination nor included in Figure 3.

Discussion.—These experiments demonstrate that fewer skin fibroblasts from the offspring of two diabetic parents grow as colonies in comparison with fibroblasts derived from normal controls. Thus, the effect of the diabetic gene(s) is evident at the cell level in fibroblasts.

The interpretation of these data depends on whether, in both types of cultures, the cells able to form colonies represent a random sample of a homogeneous population or a special fraction of a heterogeneous population. Colony formation involves the ability of a single cell to survive trypsinizing and dilution, and to attach and multiply, forming a macroscopic colony within a fixed period of time. In recent years, improvements in the techniques of handling cells and the design of culture media have increased markedly the number of fibroblasts able to form

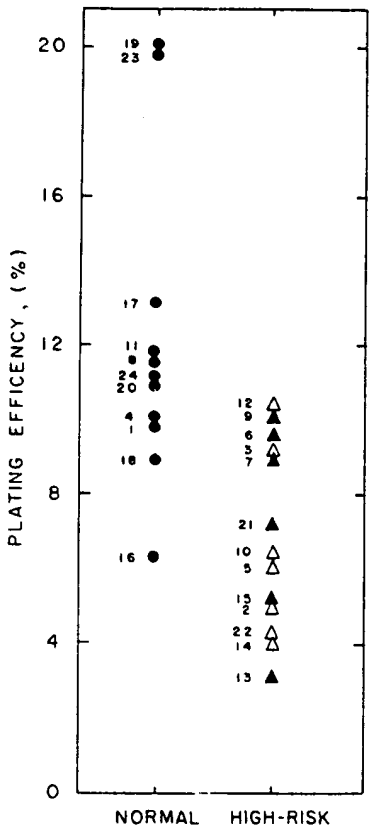

FIG. 2.—Plating efficiency of fibroblasts from control and high-risk subjects after 20 generations in culture. The data were accumulated in five experiments on different days. In each experiment, a few cultures from both groups were handled in random order and in the same medium to avoid any systematic bias. Late log-phase cells were released by trypsin from monolayer cultures. After counting and appropriate dilution, 500 cells were inoculated into each of four Petri dishes containing growth medium. The medium was replaced twice a week. Fourteen days after inoculation, dishes were stained with Giemsa. Macroscopic colonies were counted with good agreement by two people, one of whom did not know which strains were "normal" or "high risk." For each point, the SEM was too small to show. Symbols and index numbers correspond to those in Fig. 1. No explanation can be provided for the poor cloning of no. 16, who is included in the normal group. This nonobese female had normal blood glucose levels during seven glucose-tolerance tests spaced over 3 yr. However, on two such tests, serum insulin levels exceeded the mean plus 2 SD for normal controls at 3 of 10 and 1 of 10 time intervals.

colonies[21]; indeed, further improvements may either obscure or enhance differences such as those in this study. Thus, it is possible that the colony-forming cells in both groups do not represent a special fraction, but do represent an arbitrary sample of a homogeneous population. In high-risk cells, the diabetic gene(s) would then exert a universal detrimental effect. In support of this interpretation our impression is, although not quantitated, that often many of the colonies from the high-risk cultures contained less cells than those from the normal cultures. On the other hand, there is evidence for heterogeneity in fibroblast cultures, both as to enzyme content[22] and growth rate.[23, 24] Therefore, the colony-forming cells

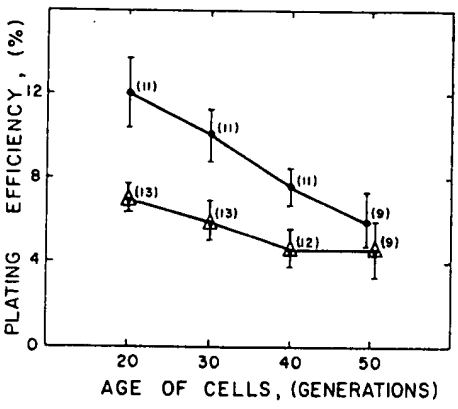

FIG. 3—Mean plating efficiencies of fibroblasts from normal controls and high-risk subjects, as a function of age *in vitro*. Procedure as in Fig. 2, using cells removed from mass culture at 20, 30, 40, and 50 generations. (The 20 generation data are from Fig. 2.) All high-risk subjects are treated as one group (△) compared to normal controls (●). Each point represents the average plating efficiency ± SEM of the cultures surviving to the generations indicated in the abscissa. The number of surviving cultures comprising the plotted mean are shown at each point.

may represent a special fraction of the cultures, this fraction being decreased in the high risk cultures.

To decide between these two interpretations, it is planned to determine the secondary plating efficiencies of cells which have been able to form colonies in a new series of experiments on control and high-risk subjects. If fibroblasts from the latter have a low efficiency on repeat testing, it is likely that they represent a random fraction of a population with universally reduced growth capacity. If they have a high secondary plating efficiency comparable to that of normals, it is likely that they represent a special fraction of cells, reduced in number in the high-risk cultures. In either case, the slopes of the curves in Figure 3 suggest that the difference in plating efficiency may be more pronounced in cells nearer to the stage of explantation. Thus, it is also planned to study the plating efficiencies of cells dissociated directly from the skin of young normal and high-risk subjects. Such experiments will concern an additional possible explanation for our data, suggested by a report that the emigration of cells from chick embryo heart explants slows with increasing age of the donor.[25] If the high-risk explants were physiologically older, fewer fibroblasts would emigrate from them and more generations would be necessary before the first passage, in comparison with normal control explants. For example, if adjustment were made for 20 extra generations in the high-risk explant, the lower (high-risk) curve in Figure 3 would become superimposed on the upper normal curve. However, this explanation is less likely because, on the average, the same amount of time (4 weeks) was used up by both groups during the explant stage and because the lifespans of both groups in mass culture were statistically indistinguishable (Fig. 1).

In any case, the main conclusion of this work is that an effect of the diabetic gene(s) is to decrease the growth capacity of some or all fibroblasts—either in the initial explant or in the cells which do or do not clone in the subsequent monolayer. Thus, a defect in diabetes has been demonstrated in cells other than the pancreatic β cell. Although the molecular basis for this is unknown, it is clear that this defect does not specifically relate to insulin production and/or release. Decreased growth capacity of cells may underlie not only the hyperglycemia of the diabetic state but may also contribute to other metabolic abnormalities and the increased vulnerability of affected individuals. Furthermore, this experimental system should permit a new approach to the inheritance of the diabetic gene(s).

The data in Figure 3 show that aging *in vitro*, as well as the diabetic gene(s), progressively decreases the plating efficiency of fibroblasts. This supplements the known effect of *in vivo* age on the *in vitro* lifespan of mass cultures (Fig. 1 and refs. 10 and 11). In conjunction with the shortened lifespans of fibroblasts from individuals with Werner's syndrome[12] and progeria,[13] these data are consistent with the concept that an aging phenotype can be demonstrated both *in vivo* and *in vitro* by a continuous decrease in viability.

Finally it may be that other inherited disorders including some that are single-gene determined and some that are polygenic will be manifested by decreased plating efficiency, which, therefore, may have usefulness as a general screening procedure.

We thank Miss Charlotte Clayton and Mrs. Babette Radner for their excellent technical assistance and Dr. Shirley Russell for her suggestions in reviewing the manuscript. We are most grateful to Miss Marguerite Burke in the laboratory of Dr. Louis Dienes for the examination for mycoplasma of these and many other cultures.

* This work was supported by Children's Bureau project no. 906 and USPHS grants CA-04670-09 and AM-09748-04 and the Joslin Diabetes Foundation, Inc., Boston, Mass. A preliminary account has been published.[26]

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